How and why *Salmonella* Typhimurium circumvents seroconversion in pigs

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INTRODUCTION

Salmonellosis is one of the most important bacterial zoonotic diseases and represents a considerable burden to humans in both developing and developed countries[1]. The consumption of contaminated pork is a major source of *Salmonella* Typhimurium infections in humans. Porcine carcass contamination is correlated with long-term *Salmonella* persistence in pigs, resulting in so called asymptomatic 'carrier pigs'. Since interference of several pathogens with the host's immune system has already been shown to be of major importance for successful long-term infection[2], *Salmonella* Typhimurium persistence in pigs could be the result of the bacterium being able to circumvent the host's cellular and/or humoral immune responses. It was the aim of the present study to examine if, how and why *Salmonella* Typhimurium escapes the porcine humoral immune system.

METHODS AND MATERIALS

As wild type we used a virulent *Salmonella* Typhimurium phage type 120/Ad strain, isolated from a pig stool sample. An *sseA* (a SPI-2 effector) deletion mutant was created as described by Datsenko and Wanner[3]. Both strains were GFP-transformed for use in flow cytometry. In an *in vivo* trial, blood samples of 17 *Salmonella*-negative piglets were collected before and 2 and 4 weeks after oral inoculation with 2 x 10^7 colony forming units (CFU) wild type *Salmonella* Typhimurium. Samples were examined for the presence of *Salmonella*-specific antibodies using a commercially available Elisa (IDEXX Laboratories). Pig antisera for opsonization experiments were raised from pigs either injected intramuscularly with formalin inactivated in Freunds incomplete adjuvant wild type *Salmonella* Typhimurium or PBS, resulting in serum with and without *Salmonella*-specific antibodies respectively.

For opsonization experiments, wild type bacteria were incubated with an equal volume of pig serum with or without *Salmonella*-specific antibodies on a shaker. Primary porcine alveolar macrophages (PAM) were collected through broncho-tracheal washes as described previously[4]. The washes were centrifuged and macrophage pellets were washed 3 times with HBSS (Gibco Life Technologies) and stored in foetal calf serum (Gibco Life Technologies) with 10% DMSO (Sigma Aldrich) in liquid nitrogen. For infection experiments, PAM were seeded in cell culture flasks (flow cytometry) or 24-well cell culture plates (intracellular survival and proliferation) for 2 h at 37 °C. PAM were inoculated with opsonized or non-opsonized wild type or Δ*sseA Salmonella* Typhimurium at MOI 10. After 30 min incubation at 37 °C, the remaining extracellular bacteria were killed by applying cell medium with 100 µg/ml gentamicin for 1 h at 37°C. Subsequently, PAM were washed and incubated for 0 h, 6h or 24 h at 37 °C in cell medium with 20 µg/ml gentamicin.

For flowcytometric analysis, 0 and 24 h after incubation, PAM were scraped off, centrifuged and incubated for 1 h on ice with a primary mouse anti-pig SLA class II DQ (1:5 dilution; AbD Serotec) or mouse anti-pig SLA class I (1:50 dilution; AbD Serotec) antibody mixture. PAM were washed and subsequently incubated for 1 h on ice and in the dark with a secondary goat anti-mouse Alexa Fluor 633 antibody mixture (1:50 dilution; Molecular Probes). After a final washing step, MHC II expression levels were measured using a FACSCanto Flow Cytometer (BD Biosciences) and analyzed with the FACSDiva Software (BD Biosciences). For determination of intracellular survival and proliferation of

Salmonella, PAM were lyzed with 1% Triton X-100 (Sigma Aldrich) 0 and 6 h after infection. Ten-fold dilutions were made, plated on BGA (International Medical Products) and incubated for 16 h at 37 °C, to assess the number of intracellular bacteria.

RESULTS

The *in vivo* experiment showed that orally inoculated pigs exhibited no seroconversion up to 4 weeks post inoculation. To explain this phenomenon, we determined *Salmonella* Typhimurium's ability to downregulate MHC II expression on PAM surface to escape the humoral immune response. Using flowcytometric analysis, we showed that MHC II but not MHC I expression was lower in infected PAM compared to uninfected PAM (Fig. 1A-B). MHC II downregulation was at least partly restored when PAM were infected with the Δ*sseA* strain (Fig. 1C-D). To determine the impact of antibodies on the interaction of *Salmonella* Typhimurium with macrophages, PAM were infected with either bacteria opsonized with porcine serum containing anti-*Salmonella* Typhimurium antibodies or with negative pig serum. Flowcytometric analysis showed that the MHC II expression level was completely restored to that of uninfected PAM when bacteria were opsonized with serum containing anti-*Salmonella* Typhimurium antibodies and not after opsonization with *Salmonella*-negative serum (Fig. 1E-F). Furthermore, intracellular proliferation of wild type *Salmonella* Typhimurium bacteria opsonized with serum containing anti-*Salmonella* Typhimurium antibodies was significantly impaired compared to that of the bacteria opsonized with negative pig serum.

DISCUSSION

Salmonella Typhimurium has already been shown to downregulate antigen presentation in murine dendritic cells[5]. In the present study, we showed that *Salmonella* Typhimurium downregulates MHC II but not MHC I expression on porcine macrophages in a SPI-2 dependent way, showing that *Salmonella* specifically targets MHC II expression and thus antibody production. Since serum antibodies inhibit MHC II downregulation by and intracellular proliferation of *Salmonella* Typhimurium in PAM, it is of great importance for the bacterium to circumvent the humoral immune response by downregulation of MHC II expression.

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TABLES AND FIGURES

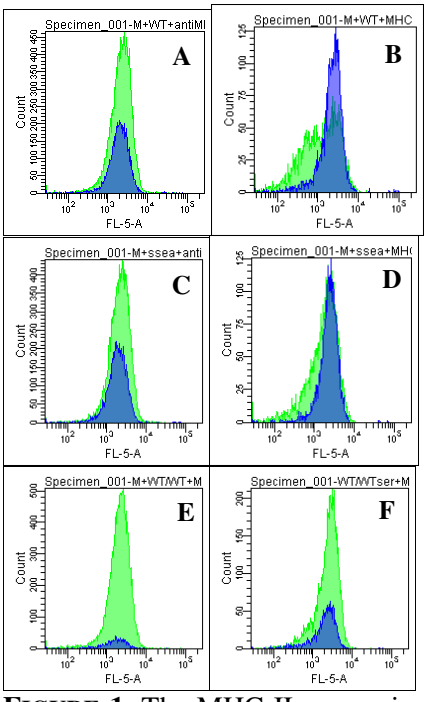

FIGURE 1. The MHC II expression level (FL-5-A) of uninfected (dark peak) compared to *Salmonella* infected (light peak) PAM. A-B. PAM 0 h and 24 h after infection with WT *Salmonella*. C-D. PAM 0 h and 24 h after infection with Δ*sseA Salmonella*. E-F. PAM 0 h and 24 h after infection with WT *Salmonella* opsonized with pig serum containing *Salmonella*-specific antibodies.